Proteolytic Cleavage of Methionyl Transfer Ribonucleic Acid Synthetase from *Bacillus stearothermophilus*: Effects on Activity and Structure†

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ABSTRACT: Methionyl-tRNA synthetase from *Bacillus stearothermophilus*, a dimer of molecular weight 2 × 85K, is converted by limited subtilisin digestion into a fully active monomeric fragment of molecular weight 64K. The reversible methionine activation reaction of these enzymes was followed through the variation of the intensity of their tryptophan fluorescence. Equilibrium and stopped-flow experiments show that the rate and mechanism for adenylate formation supported by the monomeric derivative are undistinguishable from those of each adenylating site of the native dimeric enzyme. In contrast, the rate of tRNA aminoacylation is improved upon limited proteolysis of the native enzyme. This behavior can be related to the anticooperativity of the binding of tRNA molecules to native dimeric enzyme. Accordingly, at 25 °C, the dimer might behave as a half-of-the-sites enzyme with only one active tRNA site at a time, compared to two after limited proteolysis with consequent irreversible dissociation into two 64K fragments. Another modified form of the enzyme is obtained through limited tryptic digestion. This derivative is completely devoid of activity although its molecular weight under nondenaturating conditions remains undistinguishable from that of the 64K fragment generated by subtilisin. Denaturation reveals that this tryptic derivative is composed of two subfragments with molecular weights of 33K and 29K, respectively. The same fragments may also be directly obtained through limited tryptic digestion of the subtilisic fragment. Interestingly, although trypsin treatment has abolished the activity of the enzyme, fluorescence studies demonstrate that the ATP and methionine binding sites have remained intact. It is shown that the effect of the internal cut made by trypsin into the active 64K fragment has been to considerably depress the "coupling" between the methionine and nucleotide binding sites. Finally, the rate of inactivation of the enzyme by trypsin is observed to be substantially decreased by in situ synthetized methionyl adenylate but not by tRNA. These properties and others are discussed in relation to the problem of the significance of repeating sequences and structural "domains" within the class of aminoacyl-tRNA synthetases.

Among the bacterial aminoacyl-tRNA synthetases, methionyl-tRNA synthetase from *Bacillus stearothermophilus* or *Escherichia coli* is distinguished by the fact that it is built up of two identical protomers, each containing a large proportion of repeated sequences (Koch & Bruton, 1974; Koch et al., 1974; Bruton et al., 1974). This enzyme is highly susceptible to limited proteolysis, leading to enzymatically active, irreversibly modified, fragments. For instance, the enzyme form *E. coli* is converted by trypsin into an active monomeric species (Cassio & Waller, 1971a). This conversion is accompanied by the release of three-quarters of the repeating sequences which the native protomers contained (Bruton, 1979). Structural and functional comparison of the modified enzymatic form to the intact dimer from which it originates has allowed one to reasonably speculate about the possible role of repeating sequences within the two protomers composing one native methionyl-tRNA synthetase molecule (Blanquet et al., 1979); beside the point that their integrity ensures the dimerization of the enzyme, the repeating sequences might be essential to form two structural domains which would generate a cleft as the one shown by the low-resolution tridimensional map of the modified enzyme (Zelwer et al., 1976). Despite the digestion of a major part of the repeating sequences, the limited proteolysis does not impair the activity of the *E. coli* enzyme. This may be accounted for by the fact that enough duplication has remained in order to keep intact this cleft, the structure of which may be essential for maintenance of activity.

The present study deals with the comparison to native *B. stearothermophilus* methionyl-tRNA synthetase of two derivatives resulting from different limited proteolysis. The first of these derivatives is obtained by subtilisin digestion (Koch et al., 1974; Mulvey & Fersht, 1976). Its size is very similar to that of the fragment obtained with trypsin in the case of *E. coli*. Also, the subtilisic fragment keeps its activity unimpaired with respect to that of the native enzyme from which it originates. The other derivative is obtained by limited tryptic digestion of the native *B. stearothermophilus* methionyl-tRNA synthetase. Contrary to the case of that from *E. coli*, this tryptic derivative is completely devoided of activity. Its molecular weight under nondenaturing conditions is very similar to that of the active subtilisic fragment; however, denaturation reveals the existence of two subfragments. Interestingly, although the trypsin treatment has abolished the activity of the enzyme, the ATP and methionine binding sites have remained intact. These properties and others are analyzed in structural and functional terms in relation with the question of the significance of repeating sequences in the class of aminoacyl-tRNA synthetases.

Materials and Methods

Enzymes and tRNA. Cells of *B. stearothermophilus* (strain ATCC 1518) were obtained from the Microbiological Research Establishment (Porton, U.K.). Native methionyl-tRNA synthetase from this strain was purified to homogeneity by using a standard procedure similar to that decribed in the case of the *E. coli* methionyl-tRNA synthetase (Cassio & Waller, 1971b). Modified enzymes from *B. stearothermophilus* were obtained by incubation of the purified native enzyme (2.5 mg/mL) in 0.1 M Tris-HCl buffer, pH 7.5, containing 1 mM

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2-mercaptoethanol, with bovine trypsin (DCC treated from Sigma) (at a ratio of 1:1500 w/w with respect to native enzyme) at 37 °C for 75 min or with subtilisin (subtilopeptidase A, type 8 from Sigma) (1:2000 w/w) at 37 °C for 60 min. Trypsin digestion was stopped by the addition of egg white ovomucoid trypsin inhibitor (Sigma) at a ratio of 2:1 (w/w) with respect to trypsin. Subtilisin digestion was stopped by a threefold dilution in 10 mM potassium phosphate buffer (pH 6.75) containing 10 mM 2-mercaptoethanol, followed by the addition at 0.1 mM final concentration of a phenylmethanesulfonyl fluoride solution (10 mM in butanol). The modified enzymes were further purified on hydroxylapatite as described earlier (Cassio & Waller, 1971a).

Fully active native and trypsin-modified methionyl-tRNA synthetases from *E. coli* (strain EM 20031) were obtained according to Cassio & Waller (1971a,b).

Homogeneous enzymes were stored at −20 °C in potassium phosphate (20 mM), pH 7.2, containing 10 mM 2-mercaptoethanol and 50% glycerol.

Initiator $tRNA^{Met}$ (1.4 nmol of methionine acceptance/A_{260} unit of tRNA) was obtained from *E. coli* strain EM 20031 as described by Blanquet et al. (1973).

Enzyme and tRNA molarities were calculated from their molar extinction coefficients at 280 and 260 nm, respectively.

Polyacrylamide Gel Electrophoresis. Migration of the enzymes (5–20 μg in 5–20 μL containing 25% glycerol) was carried out under native conditions at 4 °C on gradient gel slabs (4–26% acrylamide from Uniscil, U.K.) equilibrated in Tris–glycine buffer, pH 8.3. Electrophoresis under denaturating conditions was performed at room temperature on the same gradient gel slabs equilibrated in 40 mM Tris-HCl, 20 mM sodium acetate, and 2 mM EDTA, pH 7.4, containing 0.1% (w/w) sodium dodecyl sulfate. Molecular weights were determined according to Manwell (1977) after the gels were stained with Amido Schwartz 10 B (Merck) or Coomassie Brilliant Blue R (Sigma) according to Fairbanks et al. (1971).

Molar Extinction Coefficients. Specific absorbancies at 280 nm of native and modified methionyl-tRNA synthetases from *B. stearothermophilus* were obtained by refractive index measurements with the help of bovine serum albumin (Serva) and *E. coli* native or modified methionyl-tRNA synthetase as standards (Fayat et al., 1974). A constant A_{280nm} value of 1.47 ± 0.15 $cm^2\ mg^{-1}$ was found for the native, subtilisin-modified, and trypsin-modified enzymes (170 000, 64 000, and 64 000 molecular weights, respectively).

Isotopic ATP–PP_i Exchange Reaction. Initial velocities of exchange were measured at 25 °C in standard buffer (20 mM imidazole hydrochloride, pH 7.6, containing 10 mM 2-mercaptoethanol plus 0.1 mM EDTA). The assay contained catalytic amounts of the enzymes, 7 mM $MgCl_2$, and 2 mM each of L-methionine, ATP, and [^{32}P]PP (50–150 Ci/mol from the Radiochemical Center–Amersham, U.K.) (Blanquet et al., 1974).

Aminoacylation of $tRNA^{Met}$. Initial rates were measured during 20 min at 25 °C in 20 mM standard buffer (pH 7.6) containing 10 mM 2-mercaptoethanol, 35 μM [^{14}C]methionine (53 Ci/mol from the Commissariat à l'Energie Atomique, Saclay, France), 2 mM ATP, 7 mM $MgCl_2$, 150 mM KCl, 100 μM unfractionated *E. coli* tRNA containing 3.8 μM aminoacylatable $tRNA^{Met}$, and catalytic amounts of enzyme (Lawrence et al., 1973). Kinetic parameters were obtained by varying the $tRNA^{Met}$ concentration from 0.3 to 15 μM.

Guanidine Hydrochloride Treatment. The enzymes were first denatured at 25 °C in standard buffer containing 5 M guanidine hydrochloride and 10% (v/v) 2-mercaptoethanol. Reactivation of the enzymes was ensured by a 40-fold dilution in standard buffer (25 °C) in the case of the *B. stearothermophilus* enzymes (500-fold in the case of the *E. coli* enzymes).

Reactivation of the enzymes was followed by withdrawing portions at different times and measuring their initial velocity of isotopic exchange activity during 1 min after a fourfold dilution of the portions in the exchange assay. Control experiments were performed in parallel with fresh enzyme solutions directly diluted in standard buffer containing 120 or 10 mM guanidine in the case of the *B. stearothermophilus* or *E. coli* synthetases, respectively.

Fluorescence at equilibrium of the synthetases was followed at 332 nm by excitation at 295 nm, as described earlier (Blanquet et al., 1973; Hyafil et al., 1976). In the case of the titrations involving ATP or tRNA, inner effects were systematically taken into account and corrected with reference to a 60 μM tryptophan solution titrated in parallel. Parameters for titration curves were calculated by the computer using nonlinear iterative regression procedures [for review, see Kim (1970)]. Fits to the experimental data were obtained with correlation coefficients of better than 0.95.

Stopped-flow fluorescence was monitored as in Hyafil et al. (1976) with the following modifications. The amplified fluorescence signal was registered and analyzed with the help of the Nova-2 (Data General) computer equipped with 16K words (16 bits). The analogical output of the stopped-flow amplifier was stored in the computer after analogical-digital conversion (12 bits). Typically, 1000 voltage values/relaxation were stored at variable intervals of time, under the control of a programmed timer. These values were reduced to a maximum of 50 points by using polynomial least-squares regressions. Determination of the parameters for one or several averaged relaxations was then performed by the computer with the help of nonlinear iterative regression procedures.

Results

Proteolytic Modification by Subtilisin of Native B. stearothermophilus Methionyl-tRNA Synthetase. In the presence of subtilisin/enzyme ratios on the order of 1:2000 (w/w), native dimeric methionyl-tRNA synthetase (2 × 85K molecular weight) is converted within 1 h at 37 °C into a monomeric species of 64K molecular weight as shown by polyacrylamide gel electrophoresis (Figure 1). Under this treatment, the isotopic ATP–PP_i exchange activity of the enzyme sample reaches a plateau value of close to 80% of that of its initial activity (Figure 2). In contrast, the aminoacylation activity of the native enzyme is stimulated almost twofold under the effect of the limited subtilisin digestion (Figure 2).

At higher subtilisin concentrations (1:20 w/w with respect to the enzyme) the methionyl-tRNA synthetase activities of both isotopic exchange and aminoacylation are decreased to zero with corresponding digestion of the active 64K fragment.

The kinetics of modification of the enzyme by subtilisin has been followed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 3). This analysis shows that, in parallel to the active 64K species, another polypeptide of molecular weight 14 ± 2K accumulates.

Proteolytic Modification by Trypsin of Native B. stearothermophilus Methionyl-tRNA Synthetase. At trypsin/enzyme ratios on the order of 1:600 (w/w), native dimeric methionyl-tRNA synthetase is converted again into a light species. The molecular weight of this species is undistinguishable from that of the above 64K subtilisin-modified methionyl-tRNA synthetase (Figure 1). This fragment is,

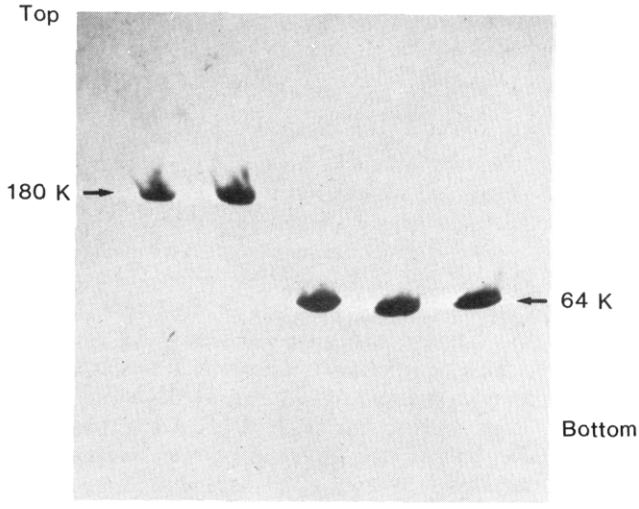


FIGURE 1: Polyacrylamide gel electrophoresis of homogeneous native and modified methionyl-tRNA synthetases from *E. coli* and *B. stearothermophilus*. From left to right: native methionyl-tRNA synthetase from *E. coli* and from *B. stearothermophilus* (molecular weight 170K each), trypsin-modified methionyl-tRNA synthetase from *E. coli* (64K), and subtilisin-modified and trypsin-modified methionyl-tRNA synthetases from *B. stearothermophilus* (64K each).

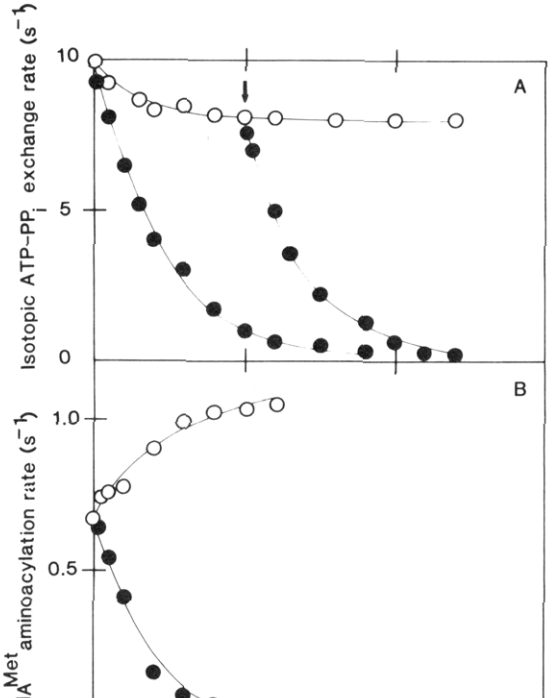


FIGURE 2: Proteolysis of native *B. stearothermophilus* methionyl-tRNA synthetase. Homogeneous methionyl-tRNA synthetase (2.5 mg/mL in 0.1 M Tris-HCl, pH 7.5, containing 1 mM 2-mercaptoethanol) was incubated at 37 °C with subtilisin (1:2000 w/w) (○) or trypsin (1:600 w/w) (●). Portions were withdrawn at different times and assayed for initial rates of isotopic ATP–[^{32}P]PP exchange (panel A) and $tRNA^{Met}$ aminoacylation (panel B). Rates on the ordinate axis were calculated with respect to initial native enzyme concentration. In panel A the incubation with subtilisin was followed by the addition (arrow) of trypsin. It was verified that the enzyme remained fully active in the absence of protease.

however, devoided of isotopic ATP–PP_i exchange activity as well as of tRNA aminoacylation activity (Figure 2). Polyacrylamide gel electrophoresis of the fragment in the presence of sodium dodecyl sulfate reveals that, in fact, it is composed of two distinct subfragments. Their molecular weights are estimated on the order of 33K and 29K, respectively (Figure 3).

Analysis on Figure 3A of the kinetics of modification shows that, in parallel to the 33K and 29K subfragments, the same 14 ± 2K polypeptide as above accumulates. Also occurring are three distinct polypeptides of molecular weights 64K, 41K, and 23K, respectively. Clearly, the 64K and 41K intermediates disappear upon completion of the trypsin modification.

Proteolytic Modification by Trypsin of Subtilisin-Modified B. stearothermophilus Methionyl-tRNA Synthetase. Limited trypsin treatment also inactivates the monomeric subtilisin-modified active fragment (Figure 2). Under this treatment, two subfragments are produced, the molecular weights of which are identical with those of the trypsin derivative obtained directly from the native enzyme. These subfragments occur without any intermediate polypeptide (Figure 3C). They remain tightly associated under nondenaturing conditions.

Reactivation of Native and Subtilisin-Modified B. stearothermophilus Methionyl-tRNA Synthetases after 5 M Guanidine Hydrochloride Treatment. After the active configuration of the enzymes was destroyed in 5 M guanidine hydrochloride, reactivation by dilution of the denaturing agent was followed as a function of time (Figure 4). Interestingly, inactivated native and subtilisin-modified *B. stearothermophilus* methionyl-tRNA synthetases recover most of their initial activity upon dilution, while, under parallel treatment, *E. coli* native and trypsin-modified synthetases appear unable to restore more than 10% of their initial activity. These behaviors as well as the rates of renaturation are not sensitive to varying the time of denaturation in the presence of guanidine from 30 to 90 min.

Finally, it must be pointed out that in this experiment the proteolyzed enzymes recover larger amounts of their initial activity than that recovered by the native enzymes from which they have originated. This is particularly clear in the case of *B. stearothermophilus* where the restored activities of the subtilisin-modified and of the native enzyme level off at 75 and 50% of their initial isotopic exchange activity prior to the guanidine treatment, respectively.

Analysis at Equilibrium of the Reaction of Methionine Activation Catalyzed by Native and Modified Methionyl-tRNA Synthetases from B. stearothermophilus. The kinetic parameters of the methionine-dependent isotopic ATP–PP_i exchange reaction were examined in the cases of native and of subtilisin-modified synthetases. From the data summarized in Table I, it clearly appears that each of the enzymes catalyzes the exchange reaction with very similar Michaelian parameters. Indeed, for each enzymic form the maximal velocities of exchange are in the ratio of their protomer number. The native enzyme composed of two subunits has a turnover number of 9.2 s^{-1}, compared to 4.6 s^{-1} in the case of the monomeric modified enzyme. Therefore, the subtilisin-modified enzyme is functionally a fully representative monomeric model of the native enzyme.

As demonstrated elsewhere, the tryptophan fluorescence of native or modified methionyl-tRNA synthetases from *E. coli* is markedly sensitive to the combinations of their various substrates (Blanquet et al., 1972, 1974; Hyafil et al., 1976). Similar behaviors are found in the cases of the native and modified enzymes from *B. stearothermophilus*. This enables us to analyze, step by step, the reactions of methionyl adenylate formation and of its reversal by pyrophosphate.

As in the case of the *E. coli* enzymes, the increase of fluorescence upon saturating methionine (10 ± 3% of the initial fluorescence) is the same for the native or for each of the two

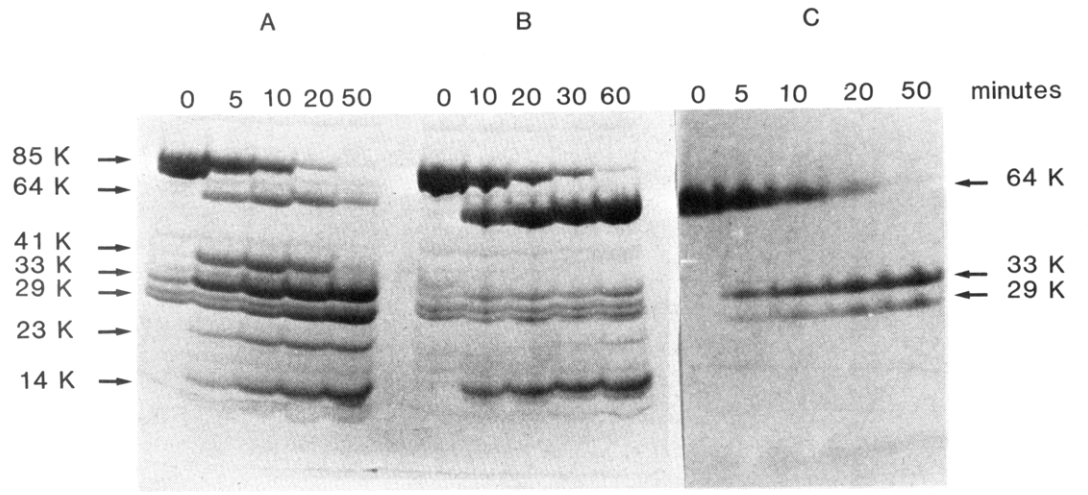


FIGURE 3: Analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of the limited proteolysis of *B. stearothermophilus* methionyl-tRNA synthetase. The native enzyme (1.6 mg/mL in 0.1 M Tris-HCl, pH 7.5, containing 1 mM 2-mercaptoethanol) was incubated at 37 °C in the presence of trypsin (1:1600 w/w) (panel A) or subtilisin (1:2000 w/w) (panel B). In panel C, the subtilisin derivative (1.5 mg/mL in the same buffer as above) was incubated at 37 °C in the presence of trypsin (1:1500 w/w). At the various times of the kinetics of digestion, which are indicated on Figure, 7-μL portions were withdrawn and the action of proteases was stopped by the addition of 1 μL of egg white ovomucoid trypsin inhibitor in 40 mM Tris-HCl, 20 mM sodium acetate, and 2 mM EDTA, pH 7.4, containing 0.1% (w/w) sodium dodecyl sulfate (final inhibitor 2:1 w/w with respect to trypsin) or of 1 μL of phenylmethanesulfonyl fluoride (0.1 mM final concentration) in 2-mercaptoethanol in the cases of trypsin and subtilisin, respectively. Portions were further denaturated for 2 min at 100 °C after the addition of 2 μL of glycerol and of 1 μL of 0.4 M Tris-HCl, 0.2 M sodium acetate, and 20 mM EDTA, pH 7.4, containing 1 M 2-mercaptoethanol, 0.1 M dithioerythritol, and 10% (w/w) sodium dodecyl sulfate, and applied on the top of the gel. Molecular weights of the various polypeptides on the gel have been estimated with reference to the following homogeneous protein markers: *E. coli* phenylalanyl-tRNA synthetase [2 × 98K + 2 × 37K (Fayat et al., 1974)]; *E. coli* native and trypsin-modified methionyl-tRNA synthetases [2 × 85K and 64K, respectively (Koch & Bruton, 1974)]; *E. coli* isoleucyl-tRNA synthetase [102K (Rainey & Holler, 1976)]; *E. coli* tyrosyl-tRNA synthetase [2 × 45K (Jakes & Fersht, 1975)]; *E. coli* tryptophanyl-tRNA synthetase [2 × 33K (Fayat et al., 1978)]; *E. coli* methionyl-tRNA transformylase [32K (Kahn et al., 1980)]; horse heart cytochrome *c* (12K from Sigma).

Table I: ATP–PP_i Isotopic Exchange Reaction Catalyzed by Native Dimeric *B. stearothermophilus* Methionyl-tRNA Synthetase and by Its Modified Derivatives[a]

	michaelian parameters			
	K_m^{AA} (μM)	K_m^{ATP} (μM)	V_m^{AA} (s^{-1})	V_m^{ATP} (s^{-1})
native	5.7 ± 1.0	12 ± 3	9.2 ± 0.3	8 ± 2
subtilisin modified	6.6 ± 1.0	12 ± 3	4.6 ± 0.2	4 ± 1
trypsin modified	6.5 ± 1.0	15 ± 5	0.11 ± 0.01	0.10 ± 0.02

[a] Initial velocities (V_i) of isotopic ATP–PP_i exchange were measured at 25 °C in 20 mM standard buffer, pH 7.6, in the presence of catalytic amounts of the various enzymes. Michaelian parameters of methionine (K_m^{AA} and V_m^{AA}) were measured in the presence of 7 mM $MgCl_2$ and 2 mM each of ATP and pyrophosphate by varying the methionine concentration in the assay from 2 to 500 μM. On the other hand, K_m^{ATP} and V_m^{ATP}, the parameters for ATP, were measured in the presence of 5 mM $MgCl_2$ and 2 mM each of methionine and pyrophosphate by varying ATP–Mg^{2+} from 5 to 200 μM. Values in the table are regressed parameters with standard errors for the reciprocal linear plots: $1/V_i = f(1/[AA])$ or $f(1/[ATP])$. The observed minor activity of the tryptic derivative corresponds to the residual active M_r 64K fragment in this enzyme preparation. It can be accounted for by 1% of the native enzyme having not been inactivated in the course of the digestion by trypsin. Such an amount is in agreement with sodium dodecyl sulfate gel analysis of the tryptic fragment where it could be distinguished as a minor band migrating at M_r 64K, the position of the active subtilisic fragment. K_m values of methionine and ATP–Mg^{2+} supporting this weak activity have been measured. These values are very identical with those of the active M_r 64K subtilisic fragment.

modified *B. stearothermophilus* enzymes (parts A, D, and G of Figure 5). Each binding isotherm fits in a unique class of binding sites with equilibrium constants which are very similar for each of the enzymes, native or modified (Table II). Remarkably, methionine binds the inactive *B. stearothermophilus* trypsin-modified enzyme with parameters which are undistinguishable from those of the native enzyme or those of the active subtilisic fragment. Therefore, despite the loss of its isotopic ATP–PP_i exchange activity, the trypsin-modified synthetase has kept intact the site for binding methionine.

ATP–Mg^{2+} can react with the E·methionine complexes of native and subtilisin-modified *B. stearothermophilus* methionyl-tRNA synthetases, thus promoting a quenching of the enzyme fluorescence as already reported in the case of the *E. coli* enzymes. In the cases of each of the native and subtilisin-modified enzymes, the final magnitude of the fluorescence at saturating ATP–Mg^{2+} is 90 ± 1% of that of the free enzyme fluorescence (parts B and E of Figure 5). This decreasing of the fluorescence intensity has been shown to correspond to enzyme·methionyl adenylate formation (Hyafil et al., 1976; Mulvey & Fersht, 1976). The isotherms of the ATP–Mg^{2+} reaction are analyzed according to Blanquet et al. (1974). This allows us to determine equilibrium constants, [E·methionine][ATP–Mg^{2+}]/([E·methionyl adenylate][PP–Mg^{2+}]), as well as active stoichiometries for adenylate formation in the cases of native and subtilisin-modified enzymes. The constants are given in Table II and are found to be similar within standard error for the two forms of the synthetase. The stoichiometries of synthetized methionyl adenylate molecules at equilibrium are respectively 2.0 ± 0.3 and 0.74 ± 0.05/mol of native or subtilisin-modified enzyme. The titration by ATP–Mg^{2+} of the enzyme·methionine complexes has also been performed in the presence of inorganic pyrophosphatase, which shifts the equilibrium toward the enzyme·methionyl adenylate species (Hyafil et al., 1976). These active titrations are shown in parts B and E of Figure 5. Two and one molecules of adenylate are formed and bound per mol of dimeric native or monomeric subtilisin-modified *B. stearothermophilus* methionyl-tRNA synthetases, respectively. It may be noted that, at saturating methionine and ATP, the fluorescence intensities of the enzymes in the presence of pyrophosphatase have values identical with that obtained in its absence. Finally, the

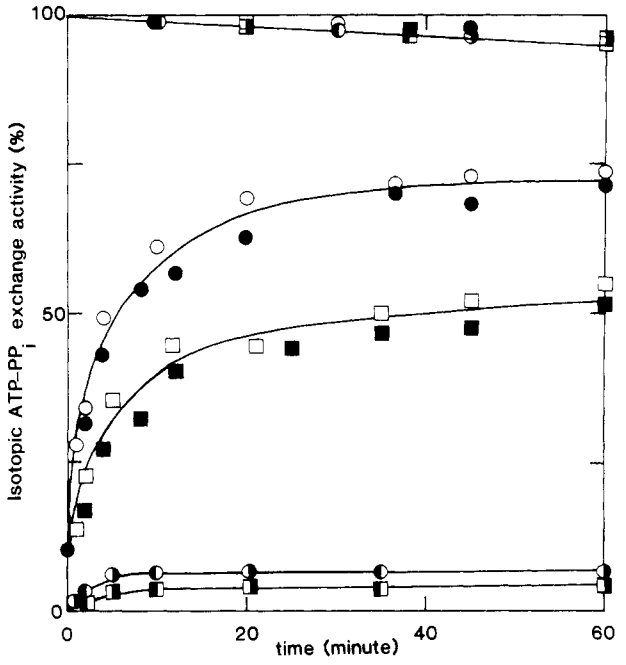


FIGURE 4: Kinetics of reactivation after guanidine treatment of native and modified methionyl-tRNA synthetases. (1) Native [60 μM (□ and ■)] or subtilisin-modified (120 μM) (○ and ●) enzymes from *B. stearothermophilus* were incubated for 30 (○ and □) or 90 min (● and ■) in 20 mM imidazole hydrochloride, pH 7.6, containing 5 M guanidine and 10% (v/v) 2-mercaptoethanol and then diluted 40-fold in the same buffer without guanidine and 2-mercaptoethanol, and the isotopic ATP–PP_i exchange activity was followed as a function of time. (2) Native (60 μM) (◨ and ◧) or trypsin-modified (120 μM) (◑ and ◐) enzymes from *E. coli* were incubated for 30 (◑ and ◨) or 90 min (◐ and ◧) in guanidine as described above and then diluted 500-fold in 20 mM imidazole hydrochloride, pH 7.6, and the isotopic ATP–PP_i exchange activity was followed as a function of time. (3) Control experiments (upper line) were performed with enzymes incubated for 30 or 90 min in 20 mM imidazole hydrochloride pH 7.6, containing only 10% (v/v) 2-mercaptoethanol and then diluted in the same buffer without 2-mercaptoethanol containing 120 or 10 mM guanidine in the case of enzymes from *B. stearothermophilus* or *E. coli*, respectively. It was also verified that concentrations of guanidine below 200 mM did not affect the activity of the enzymes under study.

fluorescence of the methionine complex of the inactive trypsin-modified enzyme remains unsensitive to the addition of excess ATP–Mg^{2+}, even in the presence of pyrophosphatase.

The reversal of methionyl adenylate formation by pyrophosphate has also been followed (parts C and F of Figure 5). In the cases of the active native and subtilisin-modified enzymes, it is accompanied by an increase in the tryptophan fluorescence of the enzymes from 90 ± 1 to 99 ± 1% at saturating pyrophosphate. There again, within the limit of accuracy of the experiments, the binding isotherms can fit in with a single set of binding sites, thus providing a unique equilibrium constant in the case of each enzyme. Values for this constant, which has been defined elsewhere (Blanquet et al., 1974), are compared in Table II. It has been verified also that in the presence of excess methionine and ATP–Mg^{2+}, the fluorescence of the inactive trypsin-modified enzyme remains unaffected by the addition of pyrophosphate (Figure 5I).

Consequently, the set of results presented in this section shows that, at equilibrium, the various reaction steps sustaining the isotopic ATP–PP_i exchange activity of the dimeric native *B. stearothermophilus* methionyl-tRNA synthetase and of its subtilisic derivative are undistinguishable. The dimeric native enzyme has two equivalent sites for adenylate formation which behave with overall thermodynamical parameters almost identical with those of the monomeric subtilisin-modified enzyme. Interestingly, the inactive trypsin-modified derivative, the fluorescence of which no longer reflects the characteristic methionyl adenylate formation, is devoided of isotopic exchange activity. The binding site for methionine has, however, remained intact on this modified enzyme.

Stopped-Flow Analysis of Methionyl Adenylate Synthesis Catalyzed by Dimeric Native B. stearothermophilus Methionyl-tRNA Synthetase or Its Monomeric Subtilisin-Modified Derivative. As emphasized in the previous section, when ATP–Mg^{2+} reacts with the enzyme·methionine complex, the tryptophan fluorescence of the enzyme is markedly decreased upon the formation of enzymatically bound methionyl adenylate. Thus, a time-dependent variation of the fluorescence can be followed with the stopped-flow apparatus.

In agreement with a previous observation by Mulvey & Fersht (1976), methionyl adenylate formation is characterized by a biexponential decrease in the fluorescence of the native enzyme (Figure 6). Biphasic kinetics are observed upon reaction of the enzyme with ATP–Mg^{2+} in the presence of excess methionine as well as upon reaction with methionine in the presence of excess ATP–Mg^{2+}. In contrast, the increase of fluorescence corresponding to reversal of the adenylate formation by pyrophosphate, can be fitted in with a single monophasic relaxation.

Mulvey & Fersht (1976) have proposed that a biphasic relaxation reflects the formation at vastly different rates of the two adenylates on the dimeric enzyme. From that, they concluded that, contrary to the case of the structurally similar *E. coli* methionyl-tRNA synthetase, the *B. stearothermophilus* methionyl-tRNA synthetase exhibited negative cooperativity in substrate binding and virtually half-of-the-sites reactivity.

In comparison with this, it has been unexpectedly found that the active monomeric subtilisin-modified *B. stearothermophilus* enzyme exhibited also biphasic kinetics identical with those obtained with the dimeric native enzyme (Figure 6). In each case of the native enzyme and of its monomeric derivative, the rate constants and amplitudes for the various relaxations have been systematically followed as a function of methionine, ATP, and pyrophosphate concentrations. From this comparison, no difference emerged which might indicate that the native enzyme has adenylating sites different from that of the monomeric fragment.

The ligand dependence of the rate constants has been analyzed with the help of a reaction scheme which has already been developed and assessed in the case of *E. coli* methionyl-tRNA synthetase (Hyafil et al., 1976). Among the relaxations with a decreasing signal, only the dominant fast exponential was considered. In fact, in the range of concentrations which could be explored here, the rate of the slow minor exponential exhibited a weak ligand dependence.

The reactions of the native and subtilisin-modified enzymes can be fitted with the parameters in Table II which are presented together with the parameters determined at equilibrium in the previous section. Clearly, each site of the native dimeric enzyme has parameters very similar to those of the site of the monomeric modified enzyme. With the possibility remaining that, under the conditions of methionyl adenylate synthesis, the subtilisic fragment had associated into dimers, the following experiment was performed. The 64K fragment (10 μM) was cochromatographied with *E. coli* isoleucyl-tRNA synthetase (molecular weight 110K) on a column of Sephadex G-200 (60 × 1 cm) equilibrated in standard buffer containing 2 mM methionine, 0.5 mM ATP, and 7 mM $MgCl_2$. The 64K enzyme was recovered at a concentration of 1 μM in a well symmetrical peak. It eluted at the expected position, at the

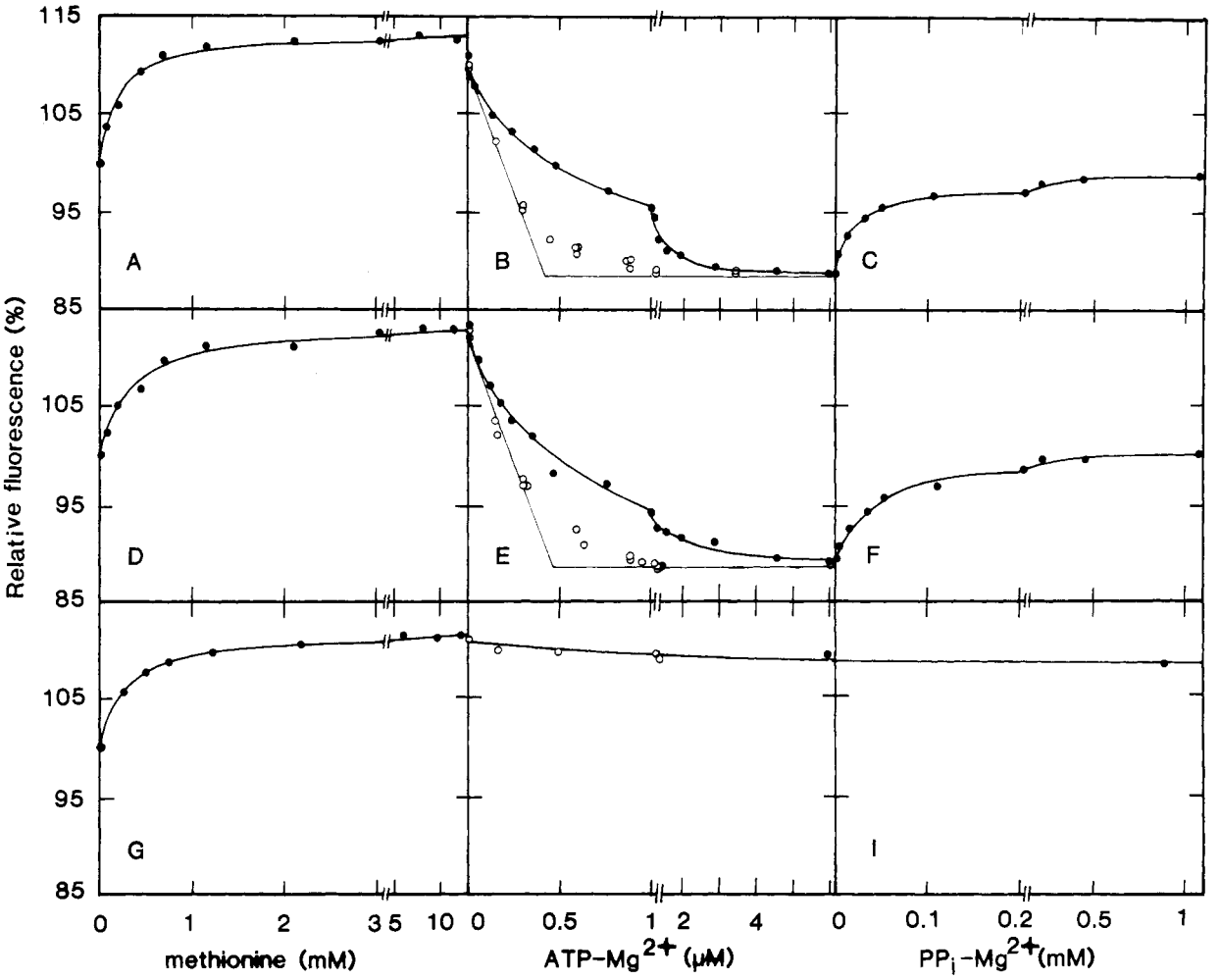


FIGURE 5: Fluorescence at equilibrium of the reactions of methionyl adenylate formation and reversal by pyrophosphate catalyzed by native (A–C), subtilisin-modified (D–F), and trypsin-modified (G–I) methionyl-tRNA synthetases from *B. stearothermophilus* at concentrations of 0.31, 0.64, and 0.82 μM, respectively. The enzymes were titrated by (1) methionine (A, D, and G), (2) ATP–Mg^{2+} in the presence of 2 mM methionine (B, E, and H) with (○) or without (●) 0.12 μg/mL *yeast* inorganic pyrophosphatase, and (3) PP–Mg^{2+} in the presence of both 2 mM methionine and 120 μM ATP–Mg^{2+} (C, F, and I). In the case of the active ATP–Mg^{2+} titration of the native enzyme·methionine complex in the presence of pyrophosphatase (panel B), the enzyme concentration was 0.21 μM. The active titrations (B and E) provide stoichiometries of adenylate per mole of enzyme at the intersection of the final fluorescence plateau with the extrapolated initial slope of the titration curve (Hyafil et al., 1976). All experiments were performed at 25 °C in 20 mM imidazole hydrochloride, pH 7.6, containing 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 7 mM $MgCl_2$. Fits according to the parameters in Table II are superimposed on the experimental values.

back of isoleucyl-tRNA synthetase. Therefore, as in the case of the equivalent *E. coli* system (Hyafil et al., 1976), it can be safely concluded that, at 25 °C, each monomer of the dimeric *B. stearothermophilus* methionyl-tRNA synthetase behaves independently, exhibiting the same parameters for the methionine activation reaction as does the monomeric subtilisin-modified enzyme.

Rate values in Table II can be compared to overall rates of isotopic ATP–PP_i exchange (Table I). Surprisingly, k_f and k_b, the forward and backward rates of the adenylation reaction, combine in order to give a rate per active site for the isotopic exchange equal to 18 s^{-1}, a value significantly higher than the rate directly measured (4.6 s^{-1}). This may reflect that all of the association–dissociation processes involved in the isotopic exchange reaction are not in rapid preequilibrium with respect to the limiting rate of the adenylation reaction. In turn, this discrepancy may also be related to the significance of this slow relaxation which is observed upon aminoacyl adenylate formation in the stopped-flow experiments. On the basis of the fact that the rate of this slow relaxation does not vary significantly with the concentration of reactants (methionine and ATP–Mg^{2+}), it may be proposed that this phenomenon corresponds to rate-limiting zero-order rearrangement of the enzyme·aminoacyladenylate complex, following the fast first-order bimolecular step corresponding to adenylate formation. Under this hypothesis, a lowered isotopic exchange rate would be explained by part of the enzyme·aminoacyl adenylate complex at equilibrium being turned aside from the rapid isotopic exchange. In that case, however, two phases should have been expected in the pyrophosphorolysis reaction. Yet the experiments failed to show this evidence; it remains that some complexity of the adenylate reversal by PP_i is indicated by the noticeable discrepancy in Table II between K_X values measured either at equilibrium (Figure 6) or from the amplitudes of the stopped-flow relaxations.

Coupled Binding of ATP–Mg^{2+} and L-*Methioninol to Native and Modified B. stearothermophilus Methionyl-tRNA Synthetases.* The coupling between ligands (i.e., the change in the dissociation constant of one ligand upon binding of another ligand) within the adenylating sites of *E. coli* aminoacyl-tRNA synthetases has recently emerged as an important factor contributing to the specificity of their amino acid activation reaction (Kosakowski & Holler, 1973; Fayat & Waller, 1974; Blanquet et al., 1975a,b; Holler et al., 1975; Fayat et al., 1977a,b). While the bindings to *E. coli* synthetases of amino acid and ATP–Mg^{2+} are weakly coupled,

Table II: Parameters for the Reactions of Methionyl Adenylate Formation and Reversal by Pyrophosphate Catalyzed by Native Dimeric *B. stearothermophilus* Methionyl-tRNA Synthetase and by Its Subtilisin-Modified Monomeric Derivative[a]

	native	subtilisin modified
analysis at equilibrium		
$[E][AA]/[E\cdot AA] = K_{AA}$ (μM)	267 ± 40	337 ± 60
$[E\cdot AA][ATP]/([E\cdot AA\sim AMP][PP]) = K_{f1}$	0.85 ± 0.07	0.42 ± 0.015
$[E\cdot AA\sim AMP][PP]/([E\cdot AA\sim AMP\cdot PP] + [E\cdot AA\cdot ATP]) = K_X$ (μM)	25 ± 5	30 ± 5
n	2.0 ± 0.3	0.74 ± 0.05
n^*	1.9 ± 0.1	0.82 ± 0.10
stopped-flow analysis		
k_f (s^{-1})	18.9 ± 2.3	20.4 ± 2.3
k_b (s^{-1})	200 ± 10	146 ± 20
$[E\cdot ATP][AA]/[E\cdot ATP\cdot AA] = K_{AA}{}^{ATP}$ (μM)	2.3 ± 5.3	3.6 ± 2.1
$[E\cdot AA][ATP]/[E\cdot ATP\cdot AA] = K_{ATP}{}^{AA}$ (μM)	10.9 ± 6.1	17.9 ± 2.4
$[E\cdot AA\sim AMP][PP]/[E\cdot AA\sim AMP\cdot PP] = K_{PP}{}^{AA\sim AMP}$ (μM)	81 ± 12	73 ± 32
K_X (μM)	4.9 ± 1.3	7.4 ± 2.6

[a] The analysis at equilibrium corresponds to the experiments presented in Figure 5. The dissociation constant of methionine, K_{AA}, is obtained from the titration curves in panels A and D of Figure 5 in the cases of the native and of the subtilisin-modified enzymes, respectively. For comparison, the K_{AA} value of the tryptic derivative is 220 ± 30 μM. The K_{f1} values are deduced from the titrations by ATP-Mg^{2+} of the enzyme·methionine complex (panels B and E of Figure 5). These experiments also provide the number of methionyl adenylate molecules, n, synthetized per mole of enzyme (Blanquet et al., 1974). n^* is the number of active sites per mole of enzyme measured in the presence of pyrophosphatase (Hyafil et al., 1976). The reversal by pyrophosphate of the enzyme saturated with methionine and ATP-Mg^{2+} (panels C and F of Figure 5) enables us to calculate K_X values, $[E\cdot AA\sim AMP][PP]/([E\cdot AA\sim AMP\cdot PP] + [E\cdot AA\cdot ATP])$, according to the reaction scheme in Blanquet et al. (1974). The stopped-flow analysis has been performed by varying (1) methionine from 5 to 500 μM in the presence of 500 μM ATP-Mg^{2+}, (2) ATP from 5 to 500 μM in the presence of 2000 μM methionine, and (3) PP-Mg^{2+} from 5 to 320 μM in the presence of both 2000 μM methionine and 500 μM ATP-Mg^{2+}. In the cases of the relaxations corresponding to adenylate formation, the kinetics were analyzed with the help of two exponentials (see Figure 6). Only the fast exponential was used for the calculation of the values in the table. The whole set of obtained rate constants was fitted to the relationship $k = k_f G^{-1} + k_b H^{-1}$, with $G = 1 + K_{ATP}{}^{AA}/[ATP] + K_{AA}{}^{ATP}/[AA] + K_{ATP}{}^{AA}K_{AA}/([AA][ATP])$ and $H = 1 + K_{PP}{}^{AA\sim AMP}/[PP]$ as assessed in Hyafil et al. (1976). k_f is the rate of reaction from $E\cdot AA\cdot ATP$ to $E\cdot AA\sim AMP\cdot PP$, while k_b is the inverse reaction. The K_X values, $[E\cdot AA\sim AMP][PP]/([E\cdot AA\sim AMP\cdot PP] + [E\cdot AA\cdot ATP])$, given in the later part of the table were derived from the amplitude analysis of the relaxations obtained upon reversal by pyrophosphate of the enzyme·methionyl adenylate complex.

if at all, prior to their conversion into adenylate, the removal of only one of the negatively charged reacting groups of these ligands (the carboxylate of the amino acid or the α-phosphate of ATP-Mg^{2+}) triggers synergistic coupling of their binding to the enzyme. For instance, in the case of *E. coli* methionyl-tRNA synthetase, it has been shown that the binding of ATP-Mg^{2+} is improved 60-fold by the presence of methioninol (an unreactive analogue of methionine which lacks the carboxylate group), while binding of methionine is increased 300-fold by previous binding to the enzyme of both adenosine and PP_i-Mg^{2+} (a combination which simulates ATP-Mg^{2+} without the reacting α-phosphate group). This coupling energy, which is no longer observed at the level of the intact enzyme·methionine·ATP-Mg^{2+} reacting complex, has been assumed to overcome the geometric and entropic requirements for aminoacyl adenylate synthesis.

In the case of *B. stearothermophilus* methionyl-tRNA synthetase, comparison of the equilibrium constants of methionine with the enzyme complexed or not by ATP-Mg^{2+} ($K_{AA}{}^{ATP}$ and K_{AA}, respectively, in Table II) indicates, at 25 °C, a positive coupling (50-fold) between the methionine and ATP-Mg^{2+} binding sites. However, on substituting methionine by methioninol, as for the *E. coli* enzyme, a much larger coupling is revealed (Figure 7). In the case of the native and subtilisin-modified *B. stearothermophilus* enzymes, the affinity of methioninol is reinforced 1000-fold by the presence of saturating ATP-Mg^{2+} and reciprocally (Table III). Thereagain, the removal of the negatively charged carboxylate function of the amino acid has revealed an uptake of free energy for the binding of ATP-Mg^{2+}, and it can be concluded that this coupling energy, which is no longer recovered as the free energy of binding in the methionine·ATP-Mg^{2+} coupling, has been expensed in order to pay for overcoming the constraints in the transient state of the adenylate reaction.

Since it has been shown further above that the inactive trypsin-modified methionyl-tRNA synthetase has kept intact its methionine binding site, it was of interest in the case of this enzyme to follow methioninol binding as a function of ATP-Mg^{2+}. The results in Figure 7 show that ATP-Mg^{2+} can couple synergistically with the binding of the amino acid analogue to the inactive tryptic fragment. However, the extent of coupling between the two sites (K_{AAol} vs. $K_{AAol}{}^{ATP}$ in Table III) is vastly depressed in comparison with the active native or subtilisin-modified enzymes. Binding of methioninol to the tryptic derivative is improved 8-fold by saturating ATP-Mg^{2+}, compared to 1000-fold in the case of the active enzyme.

These behaviors enable us to derive a binding constant of ATP-Mg^{2+} to each free enzyme (Table III). K_{ATP} constants of the same order of magnitude are found in the cases of each of the active native and subtilisin-modified synthetases as well as in the case of the inactive trypsin-modified enzyme. Despite large standard errors, these K_{ATP} constants are in agreement

Table III: Coupling between the Sites for L-Methioninol and ATP-Mg^{2+}[a]

	K_{AAol} (μM)	K_{ATP} (μM)	$K_{ATP}{}^{AAol}$ (μM)	$C = K_{AAol}/K_{AAol}{}^{ATP}$
native	590 ± 60	1350 ± 1320	1.5 ± 0.7	908
subtilisin modified	600 ± 60	1690 ± 1140	1.5 ± 0.4	1138
trypsin modified	645 ± 10	840 ± 80	110 ± 10	7.74

[a] The dissociation constant, K_d, of methioninol (AAol) to the enzymes was measured by fluorescence at 25 °C in 20 mM standard buffer (pH 7.6) containing 7 mM $MgCl_2$ and various ATP-Mg^{2+} concentrations. Values in the table with their standard errors were obtained by fitting the data in Figure 7 to the relationship $K_d = K_{AAol}(1 + [ATP]/K_{ATP})/(1 + [ATP]/K_{ATP}{}^{AAol})$, with $K_{AAol} = [E][AAol]/[E\cdot AAol]$, $K_{ATP} = [E][ATP]/[E\cdot ATP]$, $K_{ATP}{}^{AAol} = [E\cdot AAol][ATP]/[E\cdot ATP\cdot AAol]$, and $K_{AAol}{}^{ATP} = K_{AAol}K_{ATP}{}^{AAol}/K_{ATP}$. C is the coupling exerted by ATP-Mg^{2+} on methioninol binding parameters, i.e., the ratio of the dissociation constants of methioninol to the enzyme at zero and saturating concentrations of ATP-Mg^{2+}, respectively.

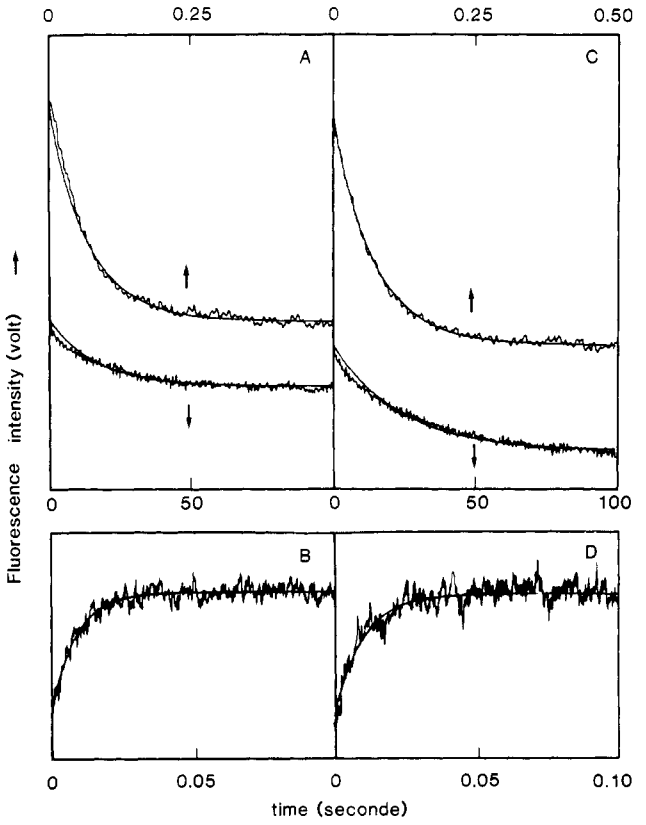


FIGURE 6: Stopped-flow kinetics for the formation of methionyl adenylate (A and C) and its reversal by pyrophosphate (B and D) catalyzed by native (A and B) and subtilisin-modified (C and D) methionyl-tRNA synthetases from *B. stearothermophilus*. Relaxations A and C (on two time scales) were obtained by 1:1 mixing of solutions of enzymes (0.47 and 0.82 μM for native and modified enzymes, respectively) containing 2 mM methionine with a solution containing 2 mM methionine and 100 μM ATP–Mg^{2+}. Initial voltages of the trace and amplitudes of the relaxations were 4.77 and −0.8 V in the case of the native enzyme and 4.85 and −0.92 V in the case of its subtilisic derivative. The rise time was 5 ms. The relaxations for methionyl adenylate formation were fitted in with the help of two exponentials, the amplitude ratios of which were 78.4 and 21.6% in the case of the native enzyme and 68.5 and 31.5% in the case of the subtilisin-modified enzyme, respectively. Relaxations B and D were obtained by 1:1 mixing of solutions of enzymes (0.5 and 1.0 μM for native and modified enzymes, respectively) containing 2 mM methionine and 500 μM ATP–Mg^{2+} with a solution containing also 2 mM methionine and 500 μM ATP–Mg^{2+} plus 160 μM PP–Mg^{2+}. Initial voltages and amplitudes were 10.66 and 0.43 V in the case of the native enzyme and 9.25 and 0.52 V in the case of the derivative. The rise time was 0.5 ms. Relaxations shown are the mean values of traces from four (A and C) or six (B and D) mixing experiments. Calculated fits are superimposed on the relaxations. All the experiments were performed at 25 °C in 20 mM imidazole hydrochloride, pH 7.6, containing 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 7 mM $MgCl_2$.

with values computed from Tables I and II according to the relationship $K_{ATP} = K_{AA}K_m^{ATP}/K_m^{AA}$ (Blanquet et al., 1974) (562 and 612 μM for native and subtilisin-modified enzymes, respectively).

Therefore, despite the loss of its activity, the tryptic derivative has also kept intact its ATP–Mg^{2+} binding site. It is tempting to assume that, although the methionine and nucleotide binding sites have remained intact, the effect of the internal cut made by trypsin into the active 64K fragment has been to depress the coupling between these sites. Consequently, the lack of activity of the tryptic derivative could be accounted for by the disappearance of this coupling energy which is required in order to constrain the substrates on their way toward the adenylate.

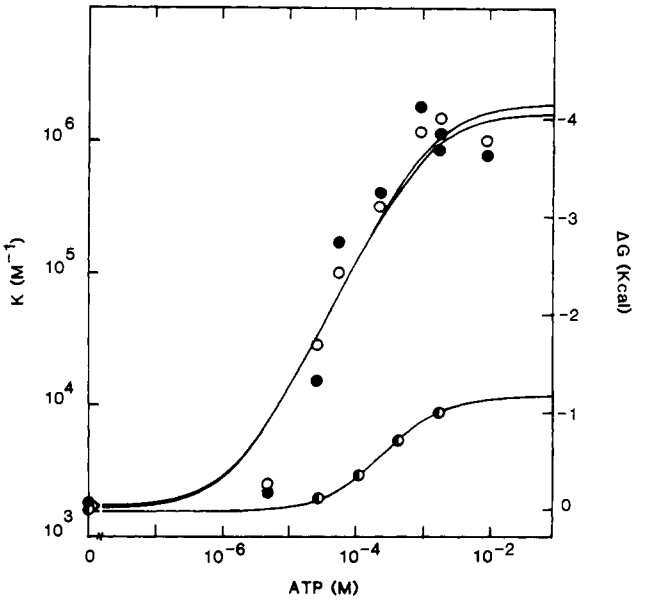


FIGURE 7: Synergistic coupling between L-methioninol and ATP–Mg^{2+} binding to native (●), subtilisin-modified (○), and trypsin-modified (◐) methionyl-tRNA synthetases from *B. stearothermophilus*. The apparent binding constant, K, of methioninol to enzymes was followed as a function of ATP–Mg^{2+} concentration. Each binding constant, K, is measured from fluorescence titration of the enzymes (0.4, 0.7, and 0.6 μM, respectively) with methioninol at the various ATP–Mg^{2+} concentrations. All the experiments were performed at 25 °C in 20 mM imidazole hydrochloride, pH 7.6, containing 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 7 mM $MgCl_2$. The ordinate on the right of the figure, ΔG, is the free energy of coupling exerted by the nucleotide on methioninol binding. Fits according to the relationship in Table III are superimposed on experimental values.

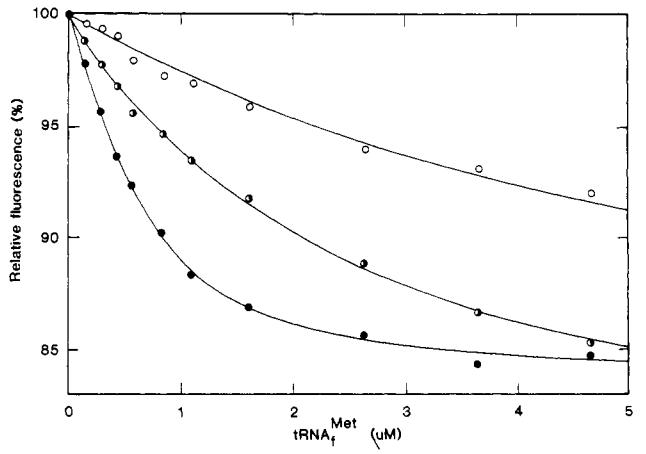


FIGURE 8: Fluorescence titration by *E. coli* $tRNA_f^{Met}$ of native (●), subtilisin-modified (◑), and trypsin-modified (○) methionyl-tRNA synthetases from *B. stearothermophilus* at concentrations of 0.57, 1.33, and 1.41 μM, respectively. All experiments were performed at 25 °C in 20 mM imidazole hydrochloride, pH 7.6, containing 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 7 mM $MgCl_2$. Fits according to the parameters in Table IV are superimposed on the data.

tRNA Binding and Aminoacylation. Binding of *E. coli* $tRNA_f^{Met}$ to *B. stearothermophilus* methionyl-tRNA synthetase and to its modified forms was followed through the quenching of tryptophan fluorescence, as already described (Blanquet et al., 1973; Dessen et al., 1978; Mulvey & Fersht, 1978). The binding isotherms of tRNA, which are shown in Figure 8, have been fitted in with the parameters summarized in Table IV. Two reaction schemes have been considered. The scheme with n independent tRNA binding sites, each with the same affinity constant, fails to give an integral stoichiometry in the case of the native enzyme. Consistent

Table IV: Binding Parameters to Native and Modified *B. stearothermophilus* Methionyl-tRNA Synthetases of *E. coli* Initiator tRNA[a]

	native	subtilisin modified	trypsin modified
K (μM^{-1})	3.7 ± 0.9 ($K_1 = 22.7 \pm 6.8, K_2 = 0.45 \pm 0.05$)	0.62 ± 0.10	0.17 ± 0.01
n	1.3 ± 0.2 ($n_1 = n_2 = 1.0 \pm 0.15$)	1	1
$\Delta\phi$ (%)	-16.6 ± 0.5 ($\Delta\phi_1 = \Delta\phi_2 = -10.0 \pm 0.3$)	-20.4 ± 1.0	-20.4

[a] Fluorescence quenching curves of Figure 8 were fitted to the following model: $E + n tRNA \rightleftharpoons E \cdot tRNA_n$, assuming n independent sites, each with an affinity constant, K. $\Delta\phi$ is the corresponding relative extent of quenching at saturating tRNA. The n values of subtilisin- and trypsin-modified enzymes as well as the $\Delta\phi$ of the latter have been fixed into the calculation. In the case of the native enzyme, the binding isotherm has also been fitted to the following model: $E + (n_1 + n_2)tRNA \rightleftharpoons E \cdot tRNA_{n_1} + n_2 tRNA \rightleftharpoons E \cdot tRNA_{n_1+n_2}$. K_1 and K_2 are the microscopic constants for each class of independent binding sites, respectively. $\Delta\phi_1$ and $\Delta\phi_2$ are the corresponding extents of quenching of fluorescence. These values, as well as n_1 and n_2, the number of active protomer molecules for each binding step, are assumed to be identical in the calculation. Values in the table are given with corresponding standard errors according to the fits.

stoichiometric parameters are better obtained with a scheme involving stepwise binding of tRNA to different classes of independent sites. From the parameters, it can be concluded that the native dimeric enzyme binds two $tRNA_f^{Met}$ molecules anticooperatively (the second tRNA binds the enzyme 50 times less strongly than the former does). The fluorescence of the enzyme is decreased by 20% at saturation of the two tRNA sites. This behavior and the parameters are very similar to those obtained by Mulvey & Fersht (1978) for the binding of the homologous $tRNA_m^{Met}$ from *B. stearothermophilus*.

The binding isotherms in Figure 8 also indicate that each of the subtilisic and tryptic 64K fragments interacts with $tRNA_f^{Met}$. Upon binding one $tRNA_f^{Met}$ molecule per mol of enzyme, the fluorescence of the monomeric subtilisin-modified enzyme is also quenched by 20%. However, the associated equilibrium constant is markedly decreased in comparison with that for the strong tRNA binding site of the native enzyme. Finally, it must be noted that the affinity of $tRNA_f^{Met}$ for the tryptic derivative is significantly lower than that for the active subtilisic derivative. Consequently, it may be assumed that the cut made by trypsin inside the active 64K fragment has also disorganized the tRNA binding site.

The kinetic parameters of $tRNA^{Met}$ in the aminoacylation reaction catalyzed by native and subtilisin-modified methionyl-tRNA synthetases were also examined. In the case of the dimeric enzyme, changes in the concentration of $tRNA^{Met}$ affect the initial rate of formation of methionyl-$tRNA^{Met}$ in an unusual manner: two sets of K_m and V values are found depending on the range of concentrations of $tRNA^{Met}$ explored. Values for K_m are 0.4 and 10 μM with corresponding V values of 0.7 and 1.8 s^{-1}, respectively. In the case of the monomeric subtilisin-modified enzyme, a unique K_m of 3.6 μM is found. It corresponds to a V value of 0.8 s^{-1}.

Anticooperativity of tRNA binding to the dimeric synthetase under the conditions of the aminoacylation reaction is susceptible to account for two sets of K_m and V values. Accordingly, in the lower range of $tRNA^{Met}$ concentration, the dimer is allowed to behave as a half-of-the-sites enzyme with only one active tRNA site at a time. In the higher range of $tRNA^{Met}$ concentration, saturation of the second active tRNA site becomes significant and the rate of aminoacylation increases up to a V value close to twice that observed in the lower range of tRNA concentration.

Finally, the validity of the subtilisin-modified enzyme as a monomeric model of the native one is well supported by comparison of its V value to that of the dimer. The turnover rate of 1 mol of the modified monomer saturated by one $tRNA^{Met}$ molecule is close to that of 1 mol of the dimer saturated by one $tRNA^{Met}$ and half that of the dimer saturated by two tRNAs. As shown above, conversion by subtilisin of the native enzyme into monomers will increase the overall rate of aminoacylation, provided that $tRNA^{Met}$ concentration in the aminoacylation assay ensures negligible binding to the weak tRNA site on the dimer but significant binding to the modified monomer.

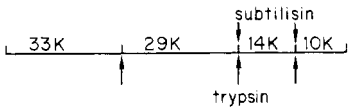
Conclusion

Methionyl-tRNA synthetases from *E. coli* and *B. stearothermophilus* are highly susceptible to limited digestion by proteases. Each of these dimeric enzymes (molecular weight 2 × 85K) is convertible into an active 64K monomeric fragment. However, contrary to the case of *E. coli*, where the native enzyme could be attacked similarly by a variety of proteolytic enzymes, including trypsin and subtilisin (Cassio & Waller, 1971a), *B. stearothermophilus* methionyl-tRNA synthetase reacts differently with each of these two proteases. The fragment obtained through subtilisin attack is fully active in isotopic ATP–PP_i exchange as well as in tRNA aminoacylation reactions. It is constituted of a unique polypeptide chain of molecular weight 64K. In turn, the attack by trypsin results in the apparition of an inactive 64K species, composed of two distinct subfragments of molecular weights 33K and 29K, respectively. These subfragments are firmly associated under the form of a molecular species undistinguishable from the active subtilisin derivative, according to polyacrylamide gel electrophoresis under nondenaturing conditions.

The digestion by trypsin of native *B. stearothermophilus* methionyl-tRNA synthetase is characterized by the appearance on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of several intermediates of molecular weights 64K, 41K and 23K, respectively. The transient character of the 64K and 41K polypeptides is clearly shown on the gel of Figure 3. This indicates that there are several routes to convert the native enzyme into the tryptic species. Taking into account that the tryptic derivative can also be obtained by limited trypsin digestion of the 64K subtilisic fragment without the occurrence of any intermediate polypeptide, we can propose the following schemes for the various proteolytic attacks. (1) In parallel to the subtilisin attack and to the conversion of the 85K native subunit into an active 64K fragment, a polypeptide of molecular weight 14K (instead of 21K) accumulates. Another polypeptide of molecular weight of close to 10K could also be distinguished provided that sufficient amounts of enzyme were applied on the top of the gel (results not shown). Therefore, within the limit of accuracy of our molecular weight estimation, two sites of recognition by subtilisin on the native subunit may be proposed. Three polypeptides are produced (64K, 14K, and 10K, respectively), one of which (10K) appears to be rather unstable in the presence of subtilisin. (2) In the case of the trypsin attack, two distinct ways of proteolysis can be distinguished. Trypsin can first liberate a 64K fragment similar to the above subtilisic derivative. This

fragment does not accumulate and is further processed into the subfragments of molecular weights 33K and 29K, respectively. In parallel to the apparition of this 64K intermediate, the 23K polypeptide shown on the gel would be formed. The intensity of staining on the gel of this polypeptide remains weak in comparison with the accumulating 29K fragment. This suggests that this 23K fragment is also further modified by trypsin. It is likely that this fragment turns into the 14K and 10K fragments which have already been observed in the case of the limited subtilisin digestion. Along the other route of proteolysis, trypsin can first cut between the 33K and 29K domains. This immediately liberates the 33K subfragment which is shown on the gel to accumulate faster than its 29K partner. Concomitantly a transient 41K polypeptide will be formed which might correspond to the above 29K polypeptide combined with either the 14K or the 10K polypeptide.

These behaviors can be summarized by the following structure for native methionyl-tRNA synthetase subunits with the various preferential attacks of proteases and the corresponding domains.



This scheme is also based upon the demonstration that subtilisin cleavage occurs on the C-terminal side (Koch et al., 1974). The respective positions of the 14K and 10K polypeptides inside the scheme can be inverted.

Considering the relative uncertainty of the molecular weight estimations, the 33K and 29K subfragments of the tryptic derivative can easily account for the entire uncleaved 64K derivative and can be the only peptides derived from it. However, the possibility remains that a relatively small peptide chain or peptides have also been formed during the trypsin attack. Nevertheless, under nondenaturing conditions, the two subfragments form a stable oligomer which comigrates with the monomeric 64K subtilisic fragment upon polyacrylamide gel electrophoresis. This suggests that the subfragments correspond to domains which are allowed to firmly interact inside either the native subunit of methionyl-tRNA synthetase or its active 64K derivative. Cleavage of a peptide bond by trypsin within the segment which associates these domains is significant enough to cause a total loss in catalytic ability. This, however, is not accompanied by the disruption of each of the active sites for methionine and ATP–Mg^{2+}. Rather, it is the coupling between sites, i.e., the amount of free energy for adenylate synthesis, which is strongly affected by the trypsin cleavage. In relation to this, we have observed that in situ synthetized methionyl adenylate decreased significantly (by 30%) the rate of inactivation of the native enzyme by trypsin (results not shown). In contrast, saturating $tRNA_f^{Met}$ had no effect on the digestion kinetics. Therefore, this coupling between the active methionine and ATP–Mg^{2+} sites of the synthetase, which is expressed under the conditions of adenylate synthesis, is enough to make the bond or bonds to be hydrolyzed less accessible to trypsin. This could occur through a conformational change at the point of attack by trypsin. A significant modification of the enzyme structure upon adenylate synthesis is indeed indicated by the associated quenching of protein fluorescence.

This model for the catalytic site of methionyl-tRNA synthetase has features similar to that proposed by Piskiewicz & Goitein (1974) in the case of the limited cleavage by trypsin of *E. coli* isoleucyl-tRNA synthetase. In that case also the limited proteolysis resulted in loss of activity of the synthetase. Two subfragments were formed which also were able to remain associated under nondenaturing conditions. Also, the rate of digestion could be substantially decreased by the presence of isoleucyl adenylate.

Aminoacyl-tRNA synthetases from various sources have been shown to undergo limited proteolysis during isolation and/or in the presence of trypsin (Rouget & Chapeville, 1971; Gros et al., 1972; Dimitrijevic, 1972; Koch et al., 1974; Epely et al., 1976; Winter et al., 1977; Kern et al., 1977; Kellermann et al., 1978a,b). It is reasonable to assume that limited proteolysis is directed toward exposed hinge regions between the compact domains of the native protein. Therefore, this class of enzymes seems to be characterized by the occurrence of these intrachain domains which have been recognized in the three-dimensional structures of numerous unrelated proteins (Schulz & Schirmer, 1979). In fact, the structures of aminoacyl-tRNA synthetases have been recently unified by the discovery that several of them exhibited extensive sequence duplication inside polypeptide chains. *E. coli* methionyl- and isoleucyl-tRNA synthetases as well as *B. stearothermophilus* methionyl-tRNA synthetase possess this feature (Bruton et al., 1974; Kula, 1973; Koch et al., 1974). It is usually accepted that the protomers which compose these enzymes may have arisen through duplication and fusion of adjacent genes each coding for one domain of the protein. For the purpose of answering the question raised by the biological significance of such "two-domain" structures, a "general symmetry recognition hypothesis" of tRNAs by the enzymes has been proposed which is based on the internal pseudosymmetry appearing in the three-dimensional structure of *yeast* $tRNA^{Phe}$ (Kim, 1975). According to the hypothesis, the pseudotwofold symmetry of an aminoacyl-tRNA synthetase is recognized by the pseudotwofold symmetry of tRNA.

The conversion by trypsin of *E. coli* methionyl-tRNA synthetase into a 64K monomeric species is accompanied by the release of three-quarters of the repeating sequences which the native protomer contained (Bruton, 1979). This, however, does not impair the activities of the enzyme (Blanquet et al., 1979, and references therein). The present study shows that the 64K fragment obtained by limited subtilisin attack of the native *B. stearothermophilus* enzyme also keeps an unimpaired activity. What is even better, its aminoacylation turnover rate is improved. The functional importance of the sequence duplication is indicated by its stabilization during evolution. If it is assumed that the role of the duplication is to ensure the specific aminoacylation of tRNA, it should be concluded that the 64K fragment, undistinguishable from the native enzyme, has kept intact enough duplication in order to sustain catalysis and specificity. Otherwise, some yet undiscovered regulatory function of the duplication should be evoked to justify its selective advantage. An interesting possibility, as in the case of bovine liver rhodanese (Ploegman et al., 1978), is that the active site of the synthetase is a pocket in between the two domains and that, inside the 64K fragment, this pocket has remained intact. Indeed, crystallographic studies of the tryptic derivative of the *E. coli* enzyme have revealed that this enzyme contained two globular parts separated by a hinge (Zelwer et al., 1976). This hypothesis fits in with the idea that, in the case of the *B. stearothermophilus* enzyme, trypsin cleaves between the two domains and gives rise to the 33K and 29K cores. Consequently, the pocket, hence the activity, is altered.

In the context of the gene duplication hypothesis, it should be assumed that each protomer of the native enzyme is composed of two domains, each with a molecular weight on the order of close to 49K. Only one of these domains would be

attacked by the protease to give the 64K protomer. This is supported by comparison of the amino-terminal residues of *B. stearothermophilus* and *E. coli* methionyl-tRNA synthetases and of their derived active fragments (Koch et al., 1974; Koch & Bruton, 1974). Therefore, it should be expected that in spite of its partial but substantial proteolysis, the domain on the side of the carboxyl end is able to maintain an active conformation. In fact, this might be better understood if the native protomer of methionyl-tRNA synthetase was composed of a minimum of three domains, as is suggested by the various proteolyzed forms of the *B. stearothermophilus* enzyme. Hence, the conversion into an active 64K fragment of the 85K native protomer would correspond to the loss of a distinct 21K domain, adjacent to the 29K domain. It may be noted that in the case of *E. coli* isoleucyl-tRNA synthetase, the trypsin attack resulted in the formation of two subfragments of molecular weights 76K and 41K, respectively (Piskiewicz & Goitein, 1974; Lee, 1974). These unequal sizes argue for a domain structure of isoleucyl-tRNA synthetase probably also more complicated than the simple twofold pseudosymmetry based on the existence of repeating sequences and on the hypothesis of gene duplication.

According to Bruton (1979), most of the repeating sequences of *E. coli* methionyl-tRNA synthetase are lost upon conversion to the active 64K fragment. This raises the question of the biological significance of such apparently unuseful repeating sequences. Due to the high stability of the *B. stearothermophilus* enzymes, it has been possible to test the idea that this excess of repeating sequences might contribute to the generation of the three-dimensional structure of the active site of the enzyme. In fact, after guanidine hydrochloride denaturation and inactivation, the 64K subtilisic fragment recovers its initial activity by dilution better than the native dimer from which it originates. Incidentally, this may reflect a simplified structure of the 64K monomer compared to the 85K subunit, for instance, two domains instead of three.

It may be useful to recall that upon the loss of the 21K fragment, which in the case of the *E. coli* enzyme has been shown to contain most of the identified repeating sequences, dimeric methionyl-tRNA synthetase irreversibly dissociates. Hence, one major characteristic of the native enzyme, anticooperative binding of $tRNA^{Met}$ (Blanquet et al., 1973, 1976; Dessen et al., 1978; Mulvey & Fersht, 1978; this work), is lost. In the case of *E. coli* synthetases, the anticooperativity of binding of tRNA has been shown to sustain a mechanism where the turnover rate of tRNA bound to the strong site of the enzyme depends on the concentration of free competing tRNA (Pingoud et al., 1975; Blanquet et al., 1976, 1979). In this work, the proteolytic uncoupling of *B. stearothermophilus* methionyl-tRNA synthetase subunits leads to a remarkable increase in the tRNA aminoacylation rate. This can be accounted for by supposing that the dimer behaves in the aminoacylation reaction as a half-of-the-sites enzyme with only one active tRNA site at a time, compared to two after the limited proteolysis and the irreversible dissociation into 64K fragments.

The establishment of oligomeric structures, consequently of kinetical regulation, might provide a rationale for the existence of the largest repeating sequences inside the protomers of bacterial aminoacyl-tRNA synthetases. Some of them, however, among which is isoleucyl-tRNA synthetase, also contain repeating sequences but are generally thought to function in vivo as monomers. In fact, evidence has been recently shown that in the presence of tRNA, monomeric aminoacyl-tRNA synthetases could aggregate and form oligomers (Zaccai et al., 1979). Therefore, the possibility remains that, besides a probable major role in fixing the active center of synthetases, the duplication feature may also be involved in catalytic regulation through the building up of yet undescribed quaternary structures inside the bacterial cell.

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Binding of Coenzyme Analogues to *Lactobacillus casei* Dihydrofolate Reductase: Binary and Ternary Complexes†

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ABSTRACT: The binding, or association, constants of $NADP^+$, NADPH, and a series of structural analogues to dihydrofolate reductase from *Lactobacillus casei* MTX/R have been determined fluorometrically. Modification of the adenine or nicotinamide rings has little effect on the binding of the oxidized coenzyme, but the thionicotinamide and acetylpyridine analogues of the reduced coenzyme bind much more weakly than NADPH itself. In the presence of the substrate, folate, or the inhibitors methotrexate or trimethoprim, the oxidized coenzymes bind appreciably more tightly to the enzyme. The magnitude of this "cooperativity", which covers a range of 1–37-fold, depends markedly on the structure of both the coenzyme and the substrate or substrate analogue; the nicotinamide ring of the coenzymes is clearly important in these effects. The binding constants of the reduced coenzymes in the presence of methotrexate or trimethoprim were too high to be measured fluorometrically. The dissociation rate constants of the coenzymes from their ternary complexes were therefore measured and compared with the values for the binary complexes reported by Dunn and co-workers [Dunn, S. M. J., Batchelor, J. G., & King, R. W. (1978) *Biochemistry 17*, 2356]. The presence of the inhibitors leads to very substantial decreases in the coenzyme dissociation rate constant—by factors of 300–2200. The binding constant of methotrexate in the ternary complex is calculated to be $\sim 1.3 \times 10^{12}\ M^{-1}$. The structural origins of the differences in binding constant and cooperative behavior of the various coenzymes and coenzyme analogues are discussed in the light of information from crystallography and NMR spectroscopy.

Dihydrofolate reductase is the target for the "antifolate" drugs such as methotrexate and trimethoprim (Hitchings & Burchall, 1965; Blakley, 1969). It is also one of the smallest known pyridine nucleotide dependent dehydrogenases, the enzyme from *Lactobacillus casei* having a molecular weight of 18 300 (Dann et al., 1976; Bitar et al., 1977). In the better known dehydrogenases, it is frequently observed that inhibitors bind substantially more tightly in the presence of coenzyme (either oxidized or reduced). Among the many examples of this are the binding of pyrazole (Theorell & Yonetani, 1963) or of fatty acids and their amides (Winer & Theorell, 1959; Woronick, 1963) to liver alcohol dehydrogenase, and the binding of carboxylic acids such as oxalate and oxamate to lactate dehydrogenase (Winer & Schwert, 1959; Kolb & Weber, 1975; Holbrook et al., 1975). Similar observations have been reported with dihydrofolate reductase (Perkins & Bertino, 1966; Williams et al., 1973a,b; Roberts et al., 1974; Birdsall et al., 1978), and this cooperativity between coenzyme and inhibitor binding may be of importance in the therapeutic action of the inhibitors.

In this and the following paper (Birdsall et al., 1980) we report measurements of the binding of the natural coenzymes and a series of structural analogues to *L. casei* dihydrofolate reductase, alone or in the presence of inhibitors. Coenzyme analogues have of course been widely employed in studies of other dehydrogenases since the early work of Kaplan and colleagues (Kaplan et al., 1956; Anderson & Kaplan, 1959), but their use with dihydrofolate reductase has been more limited. Recently Neef & Huennekens (1976) have studied the binding of etheno-$NADP^+$ and etheno-NADPH to the reductase from L1210 cells. Williams et al. (1977) have reported the activity of a number of coenzyme analogues as substrates of the enzyme, and a report from our laboratory has described the kinetics of formation of the binary complexes of various coenzyme analogues with the *L. casei* enzyme (Dunn et al., 1978). In order to interpret the differences in behavior of these coenzyme analogues, it is important to establish whether they bind to the enzyme in the same way; ^1H and ^{31}P

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